Mass Spectrometric Studies of Peptides. III.[1] Automated Determination of Amino Acid Sequences

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Abstract:

By utilizing the exact mass of the N-terminal group, the amino acid sequence can be determined from a variety of oligopeptides whose components include 14 of the known amino acids. Most derivatives giving suitable sample vapor pressure and thermal stability can be used if a mass measuring accuracy of ± 2 mmu is available. All histidine containing peptides run as the methyl esters appear to have a H atom replaced by CH_3. Automatic measurement of the spectral data plus computer calculation and interpretation of this data give promise that this method may find general use in protein research.

Key information leading to increased understanding of a number of important biological processes has been provided by the determination of the amino acid sequence in particular proteins. Present common practice for such determinations[3] involves enzymatic digestion of the protein

(3) For example, see C. H. Li, W. K. Liu, and J. S. Dixon, J. Am. Chem. Soc., 88, 2050 (1966).

followed by column and paper chromatographic separation and purification of the oligopeptide products. The sequence of amino acids in these peptides is then determined by a stepwise degradation procedure which is monitored by total hydrolysis with amino acid analysis by column chromatography, a time-consuming procedure requiring relatively large samples. An alternate source of structure information on particular oligopeptides which can utilize much smaller samples is indicated by recent publications on the mass spectra of linear and cyclic peptides and depsipeptides.[4]

(4a) E. Bricas, J. van Heijenoort, M. Barber, W. A. Wolstenholme, B. C. Das, and E. Lederer, Biochemistry, 4, 2254 (1965);

(4b) K. Heyns and H. F. Grützmacher, Ann. Chem., 669, 189 (1963);

(4c) B. J. Millard, Tetrahedron Letters, 1965, 3041;

(4d) F. Weygand, A. Prox, H. H. Fessel, and K. K. Sun, Z. F. Naturforsch., 20b, 1169 (1965);

(4e) N. S. Wulfson, V. A. Puchkov, B. V. Rozinov, A. M. Zyakin, M. M. Shemyakin, Yu. A. Ovchinnikov, A. A. Kiryuskin, and V. T. Ivanov, Tetrahedron Letters, 1965, 2793.

We have recently reported[5] a mass spectrometric method which appears to be

(5) M. Senn and F. W. McLafferty, Biochem. Biophys. Res. Commun. 23, 381 (1966).

much more generally applicable and unequivocal, and is amenable to automated calculations. This method is based on recognition of the fact that, barring rearrangements, the structure of a linear molecule is determined unequivocally by using only the possible fragments which contain one end of the chain. Thus in a hypothetical molecule A-B-C-D-E-F only six pieces

of the molecule containing part A are possible without rearrangement, and determination of these will find the sequence of the parts unequivocally. Many more combinations of these parts are possible, but their identification is not necessary for the sequence determination.

A number of methods for marking the end of the peptide chain are possible;[4a,5] high-resolution mass spectrometry appears to provide a general method.[5] Application of the latter to the determination of amino acid sequences has also been reported recently in parallel independent work by Biemann, Cone, and Webster.[6] For almost any peptide derivative of sufficient volatility, the presence of the terminal functional group makes a unique mass contribution to the exact mass of a particular fragment ion which can be combined with the predictable mass contributions of known amino acids to identify the fragments containing the terminal group. This method demands mass measurements of high accuracy on several hundred peaks in the spectrum of an average oligopeptide; such determinations are now possible on several spectra per hour using a fully automatic comparator-microdensitometer and computer techniques.[5,7] In the variety of peptide structures and derivatives examined to date it appears to be possible to extend the use of the computer to the complete solution of the amino acid sequence in oligopeptides.

(6) K. Biemann, C. Cone, and B. R. Webster, J. Am. Chem. Soc., to be published.

(7) F. W. McLafferty, Science, 151, 641 (1966).

To obtain sufficient volatility and stability both the terminal amino and carboxy groups are converted to suitable derivatives. Table I shows examples of the types of compounds from which satisfactory spectra are obtained.

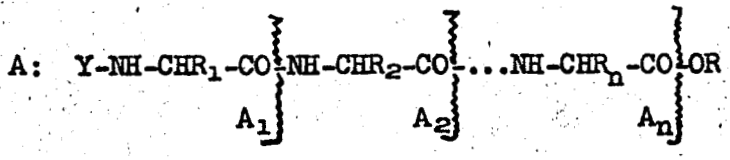
Table I

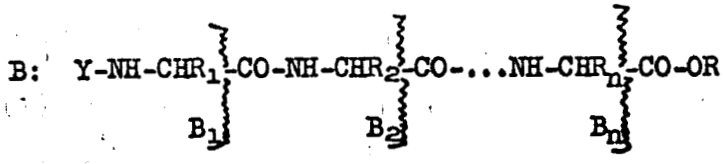
	Peptide	Temp[a]
(I)	N-trideuterioacetyl-ala-leu-ala-val-val-val methyl ester	210
(II)	N-trifluoroacetyl-leu-gly-phe methyl ester	150
(III)	N-acetyl-pro-gly-phe gly methyl ester	190
(IV)	N-trifluoroacetyl-his-pro-tyr methyl ester	200
(V)	N-trifluoroacetyl-his-met-(β-O-methyl as-) methyl ester	190
(VI)	N-trifluoroacetyl-pro-phe-his-leu methyl ester	215
(VII)	N-trideuterioacetyl-pro-phe-his-leu-leu methyl ester	235
(VIII)	N-carbobenzoxy-val-(O-t-butyl glu) methyl ester	140
(IX)	N-carbobenzoxy-ileu-(S-benzyl cys)-ser methyl ester	190
(X)	N-carbobenzoxy-val-gly-ala-leu-ala methyl ester	200

(a) Ion source temperature (°C) at which the mass spectrum was obtained.

A number of other peptides examined gave results similar to these. For some other samples suitable derivatives could not be prepared, and investigation of these is continuing. Note, however, that among the components of the peptides of Table I are 14 of the natural amino acids.

The fragmentation patterns of a variety of peptides show[4] that cleavage of the chain involves two main pathways:





These cleavages may also be accompanied by the rearrangement gain or loss

of a hydrogen atom. The relative probabilities of these reactions depend on the particular peptide and derivative. "Amine fragments" (those containing the N-terminal group Y) are generally much more abundant than "ester fragments" (those containing the -COOR group). In suitable derivatives of peptides containing only gly, ala, val, leu, pro, or phe, almost all fragment ions corresponding to scheme A and scheme B could be detected; in I-III, only the $(M - COOR)^+$ peak (B_8) in I is not found. For the histidine-containing peptides IV-VII several fragments from scheme A or B, or both, are missing.

The ester fragments from cleavages of the amide linkages in the chain decrease in abundance very rapidly with increase of the chain length retained by the fragment; ester peaks detected correspond to $H_2\overset{+}{N}=CR_n-COOR$ and $H_3\overset{+}{N}-CHR_n-COOR$.

These pathways account for only a small fraction of the detectable ions in the observed spectra, but fortunately the exact masses of these ions usually are unique. Ambiguities can arise for valine or leucine by the rearrangement loss of side-chain C_3H_8 or C_4H_8, respectively, yielding a peak which is identical in mass to the corresponding glycine-containing fragment. In some cases, such as in the spectrum of II, such rearrangement peaks are prominent; but in all spectra examined such peaks ~~were~~ are of lower intensity than the corresponding peaks due to non-rearranged ions. The occurrence of such a rearrangement is an obvious conclusion when both glycine and valine (or leucine) are indicated as the next amino acid in the sequence.

A surprising number of derivatives appear to be of a suitably unique mass if the mass measurement accuracy is better than 3 millimass units (mmu). Although N-terminal derivatives containing relatively low proportions

of hydrogen have the greatest advantage in terms of mass defects, the exact mass contributions of even CD_3CO- and, to a lesser extent, CH_3CO are sufficiently ~~unique~~ distinct in most cases. Such acetyl derivatives have the advantage of simplicity of preparation and high volatility, and give suitable fragmentation patterns for many types of peptides. However, more investigation is necessary to find optimum derivatives for particular amino acids from the point of view of both thermal stability and fragmentation patterns. For example, of the carbobenzoxy derivatives examined, the dipeptide VIII gives in low abundance the peaks predicted by both schemes A and B, while the tripeptide IX only yields the amine fragments containing the N-terminal amino acid (A_1 and B_1), although a molecular ion is produced. Pentapeptide X gives no sequence peaks corresponding to scheme A and B and no molecular ion, although other peaks in the spectrum are consistent with its structure.

Although satisfactory spectra can sometimes be obtained from peptides containing the free carboxyl group, it is more satisfactory in general to convert this to the methyl ester. The trideuteriomethyl ester derivative ~~generally~~ usually increases the ease of distinguishing the masses of the $H_2\overset{+}{N}{=}CR_n$-COOR and $H_3\overset{+}{N}$-CHR_n-COOR peaks.

General Method of Sequence Determination: Amino acid sequence is elucidated utilizing a computer program of the following general format. The first step is the identification of all peaks corresponding in mass to peaks possible from fragmentation schemes A and B. The spectrum is first checked for a peak corresponding to the sum of the exact masses of the N-derivative moiety and the glycine unit $-NHCH_2CO$, for example, 46.03722 (CD_3CO) + 57.02146 = 103.05868 (see Table II). The search is repeated

Table II

N-Derivatives	Mass
CH_3CO-	43.01839
CD_3CO--	46.03722
CF_3CO-	96.99012

Amino acid	Mass	Amino acid	Mass
gly	57.02146	asp-O-CH_3	129.04259
ala	71.03711	met	131.04048
ser	87.03203	his	137.05891
pro	97.05276	glu-O-CH_3	143.05824
val	99.06841	phe	147.06841
thr	101.04768	tyr	163.06332
leu	113.08406	try	186.07931

using combinations of the N-derivative with each of the other possible amino acids. Fragment ions corresponding to scheme B are checked by subtracting the exact mass of CO (27.99491 amu) from each of these combinations. Correspondence within experimental error of a fragment from either scheme indicates the N-terminal amino acid. If more than one amino acid is indicated as the next unit (ordinarily due to an experimental artifact or measuring error), an attempt is made to determine the next amino acid in the sequence for both of the possibilities; this almost always resolves the ambiguity. To identify the next amino acid unit of the chain, this process is repeated, with the addition of the mass (value from Table II) of the newly-identified amino acid unit to the sum of exact masses described above. This process is repeated until the search for an additional

chain member finds no suitable match for either A or B. A check is now made for the molecular ion by adding the exact mass of the ester group -OR to the mass of the identified chain, A_n. A fit then establishes the molecular size, and the identification is complete.

For some larger peptides neither a peak corresponding to the next amino acid by paths A or B nor one corresponding to the addition of -OR is found. In these cases a check for a molecular ion at higher mass is made using the sum of A_n + OR + each of the amino acid fragment masses (Table II). A similar sequence determination can be attempted from the C-terminal and using the ester fragments, making possible the structure elucidation of peptides which give no molecular ion. Some typical fragment peaks, such as $(M-H_2O)^+$, can also serve to indicate the molecular ion when it is absent. For example, if an amino acid which typically loses H_2O has been identified in the earlier part of the computer program, the peak corresponding to $(A_n + OCH_3 - H_2O)$ should be the $(M - H_2O)$ ion.

Certain peaks from other fragmentation pathways are also useful to corroborate the sequence found. The use of "dipeptide fragments" containing two amino acid units will be illustrated in the spectrum of I.

Examples.

CD_3CO-ala-leu-ala-val-val-val-OCH_3 (I). The amino acid sequence as determined by the computer program is shown in figure 1; the bar graph of

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Figure 1. Output from computer interpretation of the mass spectrum of CD_3CO-ala-leu-ala-val-val-val-OCH_3 (I).

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this spectrum at unit mass resolution is shown in figure 2. Peaks due to $(M - 15)^+$ and $(M - 42)^+$ (rearrangement loss of the valine side chain) are noteworthy. Ions corresponding to the loss of the leucine chain are at very low intensity on the photoplate, and there is no $(M - COOCH_3)^+$, the

B_8 fragment. Some peaks from pathways A and B are accompanied by peaks containing one more or one less hydrogen atom, such as fragment B_2. Masses 170, 184, and 198 have elemental compositions corresponding to the "dipeptide fragments" ala-val, ala-leu, and val-val, respectively; no peaks are found corresponding in mass to ala-ala or leu-val. The base peak in the spectrum at mass 72 corresponds to the amine fragment of valine, $C_4H_{10}N$; the corresponding leucine fragment is at m/e 86 ($C_5H_{12}N$). The two ester peaks also indicate that valine is the C-terminal amino acid.

CF_3CO-leu-gly-phe-OCH_3 (II). Table III. The spectrum shows abundant

Table III

N-Trifluoroacetyl-leu-gly-phe-OCH_3

scheme A		scheme B	scheme B
measured masses	calculated sequence	calculated masses	measured masses
	96.99012		
	113.08406 (leu)		
210.0726 [1.6]*	210.07418	182.07927	182.0781 [1.1]
	57.02146 (gly)		
267.0956 [0.0]	267.09564	239.10073	239.0991 [1.6]
	147.06841 (phe)		
414.1647 [0.7]	414.16405	386.16914	386.1682 [0.9]
	31.01839 (OCH_3)		
445.1829 [0.5]	445.18244		

* error in millimass units

A_1 and B_1 fragment peaks which could indicate a terminal CF_3CO-gly. This conclusion would then lead to finding leucine as the second amino acid. However, the computer search of all possible amino acid combinations for fragments A_1 and B_1 also finds correspondence for leucine as the N-terminal amino acid. Thus the misleading peak suggesting glycine is actually the

peak from the rearrangement loss of C_4H_8 from the leucine side chain and the computer finds the correct sequence CF_3CO-leu-gly-.

CH_3CO-Pro-gly-phe-gly-OCH_3 (III). The computer solution of the sequence is shown in figure 3. Corroboration is provided by dipeptide fragments at

Figure 3. Output from the computer interpretation of the mass spectrum of CH_3CO-pro-gly-phe-gly-OCH_3.

mass 154 for pro-gly and at mass 204 for phe-gly, and by a very intense peak at m/e 120 corresponding to the amine fragment of phenylalanine.[8]

(8) H. F. Grützmacher and K. Heyns, "Advances in Mass Spectrometry", Pergamon Press, 1966.

This spectrum shows an interesting indication of the importance of mass measuring accuracy. Small peaks are found corresponding in mass to CH_3CO-ala and CH_3CO-ala-pro with errors of 4 and 6 mmu, respectively. These would have caused considerable confusion if the error limits of the exact mass determination has not been substantially smaller than these values. Use of some other derivative such as CD_3CO- in place of CH_3CO- generally decreases the possibility of such ambiguities.

Histidine-containing Peptides. The spectra of all of the oligopeptides containing histidine, IV-VII, exhibit anomolous peaks at 14 mass units above the molecular weight. (Only IV gives a peak corresponding in mass to the expected molecular ion.) Exact mass measurements show that this difference corresponds to CH_2, indicating the replacement of a hydrogen atom by a methyl group. To ascertain the location of this postulated methyl group, the sequence determination was also made searching for masses corresponding to CH_2 above the key fragments of sequences A and B. The results, shown in Table IV, indicate that the extra methyl group is located on the

Table IV

IV	V	VI	VII	Fragment
234.0505 (1.5) his	234.0476 (1.4) his	194.0445 (1.6) pro	143.0888 (1.1) pro	A_1
248.0657 (1.9)	248.0642 (0.4)			$A_1 + CH_2$
206.0534 (0.7) (his)	206.0523 (1.8) (his)	166.0484 (0.5) (pro)	115.0952 (0.2) (pro)	B_1
220.0694 (0.6)	220.0678 (1.9)			$B_1 + CH_2$
331.1003 (1.5) pro	*	341.1114 (0.1) phe	290.1568 (1.5) phe	A_2
345.1157 (1.7)	*			$A_2 + CH_2$
303.1059 (1.0) (pro)	337.0965 (1.9) met	313.1183 (1.9) (phe)	262.1640 (0.5) (phe)	B_2
317.1229 (0.4)	351.1083 (1.9)			$B_2 + CH_2$
*	*	*	*	A_3
*	508.1482 (0.5) asp OCH_3	*	441.2341 (1.2) his	$A_3 + CH_2$
*	*	450.1772 (2.0) his	*	B_3
*	*	464.1929 (1.9)	413.2364 (1.6) (his)	$B_3 + CH_2$
		*	*	A_4
		*	*	$A_4 + CH_2$
		*	*	B_4
		*	526.3202 (1.8) leu	$B_4 + CH_2$
			*	A_5
			*	$A_5 + CH_2$
			*	B_5
			*	$B_5 + CH_2$
525.1848 (1.3) tyr	*	*	*	M^+
539.1995 (0.4)	539.1645 (1.6)	636.2880 (0.3) leu	698.4178 (1.6) leu	$M^+ + CH_2$

* No peak found corresponding to this fr

histidine moiety. The abundances of the sequence peaks containing the non-methylated histidine are too low to be detected in many cases.

These anomolous peaks may arise from some sort of a transmethylation which occurs on heating the samples in the mass spectrometer; similar artifacts have been observed.[9] Alternatively, the esterification to form the

(9) D. W. Thomas and K. Biemann, J. Am. Chem. Soc., 87, 5447 (1965).

methyl ester may also have alkylated a nitrogen atom on the histidine ring. Further investigation to elucidate this anomaly is currently in progress. However, the effect does not appear to be unique to our esterification procedure, as compound IV was supplied to us as the methyl ester. Esterification of compound V was repeated using the same procedure, but with the substitution of CD_3OD for CH_3OH. The B_3 sequence peak at mass 464 (Y-prophe-his) is increased in mass to correspond to the trideuterio species (error 2.2 mmu), and the $(M + 14)^+$ at mass 642 is increased in mass to correspond to the hexadeuterio species (error 0.4 mmu).

As an example of the value of high resolution, compound V gives a substantial peak at m/e 465, whose nominal mass could indicate the expected CF_3CO-his-met-aspOCH_3- (no extra methyl group on the histidine). However, the exact mass measurement of the peak disagrees with that expected for this ion by 14 mmu; the elemental composition actually corresponds to the loss of C_3H_6S from the molecular ion of the peptide containing the additional CH_2.

Preparation of Peptide Derivatives.

Difficulty was encountered in early derivative preparations with discoloration of derivatives, thermal decomposition of samples in the ion source, and poor reproducibility of spectra. Most of these difficulties were overcome by using purified reagents and solvents, and by avoiding any

contact with metal or dust particles.

Esterification. Approximately 0.1-0.6 micromoles of the peptide is dissolved in 40 ± 10 µl of 2-2.5 N HCl in abs. CH_3OH in a reaction flask made by blowing a 6 mm bulb in the end of a 2 mm capillary melting point tube. The top of the capillary is sealed and allowed to stand overnight at room temperature. The capillary then is opened and the HCl/CH_3OH evaporated in vacuo.

N-Acylation. The residue is dissolved in 40 ± 10 µl of the following: 1:1 acetic acid/acetic anhydride for the preparation of N-acetyl derivatives; CD_3COOH[10]$/CH_2Cl_2$ for N-trideuterioacetyl derivatives; and 1:2 CF_3COOH/

(10) From Merck, Sharpe and Dohme of Canada.

$(CH_3CO)_2O$ for N-trifluoroacetyl derivatives. The solution is transferred to an unmodified melting point capillary, sealed, and allowed to stand 4-6 hours at room temperature. After the capillary is opened the reagents are evaporated off in vacuo.

After evaporation, the product generally is distributed over the sides as well as the bottom of capillary tube. Cutting the capillary tube can usually give two parts, each with sufficient sample for a mass spectrum.

This sample tube is directly introduced into the ion source of the CEC 21-110 high-resolution mass spectrometer of Mattauch-Herzog geometry. The ion source temperatures necessary for adequate volatilization depend on the nature of the sample (see Table I). Thermal decomposition can be a problem at higher molecular weights; this can usually be detected by recording a separate spectrum at an increased temperature. Using a resolving power of roughly 12,000, spectra are recorded directly on a photoplate with perfluorokerosene as an internal mass standard. Automatic measurement

of ion line profiles is done with a Grant-Datex microdensitometer-comparator[7]

(11) F. W. McLafferty, Science, 151, 641 (1966).

with computer calculations of exact masses to an error limit of ± 2.5 mmu.

Conclusions.

A great deal needs to be done before this technique can be used as a routine tool in amino acid sequence determinations, but the prospects are promising. At present the method appears to be applicable to the majority of amino acids, and is capable of giving information not previously possible, such as the presence of methylated histidine. Sample requirements are now well below 0.1 micromoles, and current work indicates that this can be substantially reduced. The classical sequence determination of an oligopeptide requiring a number of degradations and amino acid analyses can be replaced in this scheme by one mass spectral determination, and the mass spectral measurements and calculations appear to be amenable to complete automation. Our current research program in this area includes a search for suitable derivatives of peptides containing the remaining common amino acids, use of other features of the mass spectrum and use of the mass spectra of other derivatives as corroborative evidence for the sequence found, and application of these techniques to completely unknown oligopeptides isolated from protein hydrolysates.

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Angeles; B. Witkop, National Institutes of Health; and to the National Institutes of Health (GM 12755) and to the National Aeronautics and Space Administration (NGR 15-005-021) for generous financial report.

1 AMINO ACID SEQUENCE ANALYSIS

-SAMPLE NUMBER 66-2

N-TRIDEUTEROACETYL METHYL ESTER

- THE FOLLOWING ARE THE SEQUENCE PEAKS FOUND IN THIS PEPTIDE

SEQUENCE IDENTITY	FOUND	CALC	ERROR
A-1 ALA	89.079700	89.079410	-0.29
B-1 ALA	117.073170	117.074321	1.15
A-2 LEU	202.164000	202.163470	-0.53
B-2 LEU	230.158401	230.158380	-0.02
A-3 ALA	273.201201	273.200500	-0.70
B-3 ALA	301.196100	301.195491	-0.61
A-4 VAL	372.267500	372.268990	1.49
B-4 VAL	400.264310	400.263900	-0.41
A-5 VAL	471.336600	471.337400	0.80
B-5 VAL	499.333301	499.332310	-0.99
B-6 VAL	598.402000	598.400721	-1.28
MOL-ION	629.421000	629.419110	-1.89

- AMINO ACID SEQUENCE IN THIS PEPTIDE IS

*(D)AC-ALA *LEU *ALA *VAL *VAL *VAL*

Figure 1

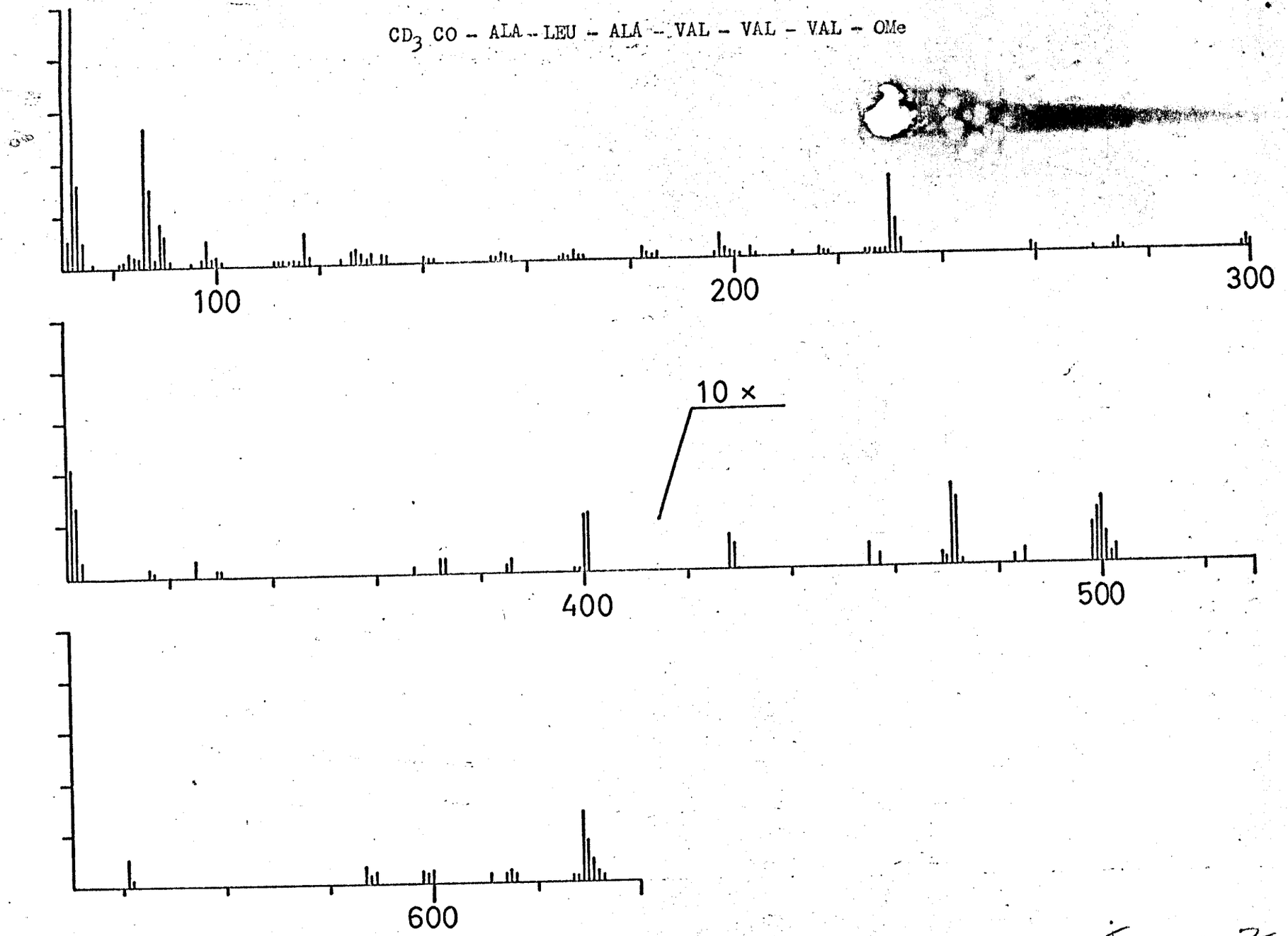

Figure 2

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Printer: Abscissa – m/e
Ordinates – Relative Abundance

1 AMINO ACID SEQUENCE ANALYSIS

--SAMPLE NUMBER 66--1

N-ACETYL METHYL ESTER

- THE FOLLOWING ARE THE SEQUENCE PEAKS FOUND IN THIS PEPTIDE

SEQUENCE IDENTITY	FOUND	CALC	ERROR
A--1 PRO	112.074300	112.076240	1.94
B--1 PRO	140.071199	140.071150	-0.05
A--2 GLY	169.095800	169.097748	1.95
B--2 GLY	197.090799	197.092659	1.86
A--3 PHE	316.164799	316.164299	-0.50
B--3 PHE	344.160500	344.159210	-1.29
A--4 GLY	373.189301	373.187046	-2.25
B--4 GLY	401.182598	401.181957	-0.64
MOL-ION	432.200401	432.200985	0.58

- AMINO ACID SEQUENCE IN THIS PEPTIDE IS

* AC--PRO *GLY *PHE *GLY *

Figure 3